Ventilatory and carotid body chemoreceptor responses to purinergic P2X receptor antagonists in newborn rats.

Lalah M. Niane¹, David F. Donnelly², Vincent Joseph¹ and Aida Bairam*.

¹Unité de recherche en périnatologie, Centre Hospitalier Universitaire de Québec, Hôpital Saint-François d’Assise, Département de Pédiatrie, Université Laval, Québec, Canada, ²Department of Pediatrics, Yale University School of Medicine, New Haven, CT, USA.

Running title: Purinoreceptors P2X and newborn carotid body

*Corresponding author:
Aida Bairam, MD, Ph.D.
Centre de Recherche, D0-717
Hôpital Saint-François d’Assise
10, rue de l’Espinay
Québec (Qc), Canada, G1L 3L5
Tel: (418) 525 4444 (4402); Fax: (418) 525 4195
e.mail: aida.bairam@crsfa.ulaval.ca
Abstract

Adenosine triphosphate (ATP), acting through purinergic P2X receptors, has been shown to stimulate ventilation and increase carotid body chemoreceptor activity in adult rats. However, its role during postnatal development of the ventilatory response to hypoxia is yet unknown. Using whole-body plethysmography, we measured ventilation in normoxia and in moderate hypoxia (12% FiO₂, 20 minutes) before and after intraperitoneal injection of suramin (P2X₂ and P2X₃ receptor antagonist, 40 mg/kg) in 4-, 7-, 12- and 21-day-old rats. Suramin reduced baseline breathing (~20%) and the response to hypoxia (~30%) in all rats with a relatively constant effect across ages. We then tested the effect of the specific P2X₃ antagonist, A317491 (150 mg/kg) in rats aged 4, 7 and 21 days. As with suramin, A317491 reduced baseline ventilation (~55%) and the hypoxic response (~40%) at all ages studied. Single-unit carotid body chemoreceptor activity was recorded in vitro in 4-, 7- and 21-day-old rats. Suramin (100 µM) and A317491 (10 µM) significantly depressed the sinus nerve chemosensory discharge rate (~80%) in normoxia (PO₂ ~150 mmHg) and hypoxia (PO₂ ~60 mmHg), and this decrease was constant across ages. We conclude that in newborn rats, P2X purinergic receptors are involved in the regulation of breathing under basal and hypoxic condition, and P2X₃-containing receptors play a major role in carotid body function. However, these effects are not age-dependent within the age range studied.

Key words: Development, hypoxia, carotid body, P2X receptors, ventilation.
Introduction

Carotid body chemoreceptors respond to a decrease in arterial oxygen saturation by increasing action potential (AP) activity in sinus nerve afferent fibers. These fibers terminate in the brainstem, and their stimulation enhances respiratory drive and sympathetic activity. Chemoreceptors undergo significant postnatal maturation. In rats, the chemoreceptor action potential response to a given level of hypoxia is weak at birth and matures to adult levels over the first three weeks of life (25). In addition, the respiratory response to hypoxia is biphasic in newborn mammals, with an initial increase in ventilation, mediated by the carotid bodies, followed by a decrease mediated by central inhibitory mechanisms and a reduction of metabolic rate (7, 17). The two phases involve different neurotransmitters which may show an age-dependence (2, 7).

Although the mechanism by which hypoxia is transduced by the carotid body to increased AP firing frequency is incompletely understood, recent data have implicated a major role of ATP acting through purinergic receptors in the adult cat, rat and mouse (20, 33, 46). For instance, intracarotid injection of ATP to anesthetized cats increases ventilation and stimulates carotid sinus nerve discharge rate (36). In addition, similar effects are observed with ATP analogues and blocked by the ATP receptor antagonist suramin (37). Pharmacological experiments based on a co-culture of rat carotid body chemoreceptor cells and sensory petrosal ganglion neurons provided evidence that the effects of ATP are mainly related to activation of postsynaptic P2X2- and P2X3-containing purinergic receptors (47, 48). Furthermore, RT-PCR and immunofluorescence studies confirmed the presence of P2X receptor subunits in chemoreceptor afferent neurons (3, 21, 34, 48). Finally, the ventilatory and carotid sinus nerve responses to hypoxia were significantly decreased in mice lacking P2X2 receptors and further reduced in P2X2/3 double knockout mice (38).

By contrast, little is known regarding the contribution of the purinergic system to maturation of hypoxic chemosensitivity in the carotid body. This maturational process involves several aspects of carotid body development,
including neurogenesis (44), establishment of synapses (9), expression of ion channels (26), and developmental changes of key neurotransmitters, such as dopaminergic and cholinergic systems. These developmental changes typically occur during a period overlapping late fetal development and the first three weeks following birth in rat pups (2, 11, 31). We recently reported that the protein expression level of P2X$_2$ and P2X$_3$ receptors remains stable during postnatal development in the carotid body of cats (3). However, their expression decreases in the petrosal ganglion; expression levels decrease by 50% in 2-month-old and adult cats compared with 1- and 15-day-old cats (3). These observations led to the hypothesis that functional purinergic signaling in the carotid body changes during development and contributes to post-natal changes in the carotid body response to hypoxia. Accordingly, we designed the present study to test this hypothesis using whole-body plethysmography and in vitro recordings of chemosensory activity in developing rats.
Material and methods

Ethics.

We used male and female Sprague-Dawley rats between postnatal (P) days 4 and 21 to conduct our studies in two different laboratories. In vivo recordings of minute ventilation in normoxia and hypoxia were performed at the Centre de Recherche du Centre Hospitalier Universitaire de Québec (CRCHUQ) after approval by the Animal Care Committee at Laval University in accordance with the Canadian Council on Animal Care guidelines. Recordings of chemoreceptor activity using the in vitro preparation were performed at Yale University School of Medicine and approved by the Institutional Animal Experimentation and Use Committee.

In vivo studies

Ventilatory and metabolic recordings

Animals. All animals used were obtained from fifteen litters by mating virgin female and male rats purchased from Charles River (St. Constant, Québec). Rats were supplied with food and water ad libitum and maintained under standard laboratory conditions (21°C, 12:12 h dark-light cycle: lights on at 07:00 and off at 19:00). Litters were culled to 12 pups on postnatal day 1 with an equal number of each sex when possible.

Measurements. Respiratory and metabolic parameters were recorded in unrestrained male and female rat pups at P4 (n=35), P7 (n=31), P12 (n=21) and P21 (n=41) using whole-body plethysmography (Emka Technologies, Paris, France) as previously described (23, 31). Using a temperature control system (Physitemp, Clifton, NJ, USA), the temperature inside the plethysmograph was fixed at thermoneutrality for each age studied: 32°C for P4 and P7 and 30°C for P12. Relative humidity was continuously monitored from the outflowing air stream with a high precision water vapor pressure analyzer (RH-300 Sable Systems International). The inner volume of the plethysmography chamber was 0.22 liter
for P4 and P7 pups and 0.75 liter for P12 and P21 pups. Calibration of the 
respiratory signal was performed by injection of 0.5 or 1 ml of air (according to the 
size of the chamber), and tidal volume (V_T) was calculated from the pressure 
development by IOX software (Emka Technologies, Paris, France). Oxygen and CO_2 
analyzers were calibrated following the manufacturer’s instructions (AEI 
Technologies; Naperville, IL, USA). Due to the small size of young rats, body 
temperature was measured orally in P4 and P7 rats and rectally in P12 and P21 
rats using a thermocouple for small rodents (Harvard, Holliston, MA, USA) before 
and at the end of the recording period. The gas flow through the plethysmograph 
was set at 0.1 L/min (P4 and P7) or 0.2 L/min (P12 and P21), and measured 
continuously with a mass flowmeter (4140; TSI, Shoreview, MN). Using a dual 
channel analyzer for oxygen and CO_2 (AEI Technologies; Naperville, IL, USA), 
respiratory gases (oxygen and carbon dioxide) were continuously measured 
during the entire recording time and used later to calculate oxygen consumption 
and CO_2 production. Calibration was performed per the manufacturer’s 
specification using a one-point or two-point method with certified gases. 
Respiratory frequency (fR) and V_T were recorded from the plethysmograph signal. 
V_T was calculated based on barometric pressure, room and body temperature, 
and humidity (BTPS) using the Bartlett and Tenney equation (5). Minute 
ventilation was calculated as \( \dot{V}_E = fR \times V_T \). Oxygen consumption was calculated 
as \( \dot{V}_O_2 = \text{flow} \times (O_{2in} - O_{2out}) - (O_{2out} \times (CO_{2out} - CO_{2in})/(1 - O_{2out}) \) and CO_2 production 
as \( \dot{V}CO_2 = \text{flow} \times (CO_{2out} - CO_{2in}) - (CO_{2out} \times (O_{2in} - O_{2out})/(1 - CO_{2out}) \), corrected to 
Standard Temperature and Pressure for Dry (STPD) conditions. These values 
were then used for calculation of the ventilatory equivalent for oxygen (\( \dot{V}_E/\dot{V}_O_2 \)) 
and carbon dioxide (\( \dot{V}_E/\dot{V}CO_2 \)) (1, 17, 30). \( V_T, \dot{V}_O_2 \) and \( \dot{V}CO_2 \) were expressed 
per 100 g body weight.

**Drugs.** All drugs were purchased from Sigma Aldrich Canada (Oakville, ON, 
Canada). Because selective P2X_2 receptor antagonists are not commercially 
available, we used the non-specific purinergic P2X receptor antagonist suramin
and the selective P2X3 receptor antagonist A317491 (22), which acts on both homomeric P2X3- and heteromeric P2X2/3-containing receptors. Each drug was dissolved in sterile saline and injected 30 minutes before starting the recordings. Based on dose-response studies performed in 19- to 21-day-old rats (see below), we administered suramin and A317491 at doses of 40 mg/kg and 150 mg/kg, respectively. The choice of a 30-minutes delay was based on association constant data from the literature (22, 28). For in vitro studies, suramin was added to the perfusion solution at 100 µM, as previously used to assess the chemoreceptor activity in adult rats (21). A317491 was used at a concentration of 10 µM based on the affinity constant measured for recombinant and native rat P2X3 receptors (22).

**Ventilatory dose-response curve for suramin and A317491 in 19-21 day old rats.** Because there are no data in the literature concerning the ventilatory effects of suramin or A317491 on young rats, we first determined the ventilatory dose response curve for each drug in normoxia (FiO2 = 21%) and in response to hypoxia (FiO2 = 12%) in 19- to 21-day-old rats (data not shown). A total of 55 rats from 6 litters were used for these preliminary studies. Four doses of suramin were tested (20, 40, 80 and 100 mg/kg, injected intraperitoneally [IP], 6 rats/dose) and compared with vehicle (saline injection, n = 10 rats). Compared with vehicle, suramin had no effect on ventilation at 20 mg/kg but decreased minute ventilation to similar levels at 40, 80 and 100 mg/kg in normoxia and hypoxia. The magnitude of this decrease was approximately 15% and 22% in normoxia and hypoxia, respectively. Accordingly, a dose of 40 mg/kg was chosen for the experimental series. For the selective P2X3 containing receptor antagonist A317491, four doses were tested: 30 mg/kg (n=3), 100 mg/kg (n=3), 150 mg/kg (n=6) and 300 mg/kg (n=3) injected IP and compared with vehicle (n=6). As with suramin, A317491 reduced minute ventilation in normoxia and hypoxia at 150 and 300 mg/kg. Each of these doses decreased minute ventilation approximately 42% in normoxia and 45% in hypoxia relative to vehicle. Accordingly, the dose of 150 mg/kg was used for the experimental series. We used both males and females for the dose-
response study. Because we found no sex-specific effect, we used male and female rats for recordings throughout the study, but we did not perform further sex-specific analyses.

**Experimental protocol.** To avoid pup- and litter-specific effects, rats from each litter were weighed and randomly assigned to one of three groups: vehicle (1 ml/kg, IP saline injection), suramin (40 mg/kg), or A317491 (150 mg/kg). Treatments were given 30 minutes before the start of the experiment. Each rat was used for only one experiment to avoid repetitive drug/hypoxic exposures. After measuring each rat’s body temperature, it was returned to the plethysmograph. Once it laid quiet, measurements were started under normoxia (FiO₂ = 21%) for 10 minutes. Nitrogen was mixed with air at the gas inlet to produce the desired chamber O₂ pressure for hypoxia (FiO₂ = 12%), which generally occurred within 1 minute and was stable during the 20 minutes recording period. All recordings were performed under poikilocapnic conditions.

**In vitro studies:**

**Carotid body chemoreceptor activity recordings**

**Animals.** Dams with their 1-day-old pups were purchased and housed in Yale University animal care facilities under standard conditions (light-dark cycle 12:12 h, food and water *ad libitum*). Male and female rat pups aged P3-4 (n=11), P7-8 (n=15) and P16-21 (n=25) from five different litters were used. For all *in vitro* experiments, each rat was only used for one recording (i.e., one carotid body/pup/experiment).

**Single-unit chemoreceptor preparation.** Each rat was deeply anaesthetized with 100% CO₂, then decapitated and the carotid body/carotid sinus nerve/petrosal ganglion complex was harvested *en bloc* under surgical microscopy as previously detailed (13, 15). The connective tissue was partially digested with a mixture of collagenase type P (1 mg/ml, Roche Diagnostics,
Mannheim, Germany) and protease type IX (0.2 mg/ml, Sigma Aldrich, St Louis, MO) in Ringer’s buffer solution (in mM: 120 NaCl, 5 KCl, 2 CaCl₂, 1Na₂HPO₄, 24 NaHCO₃, 1 MgSO₄ and 5 glucose, pH=7.4) bubbled with a mixture of 95% O₂ and 5% CO₂.

After cleaning the connective tissue, the complex was transferred to the recording chamber (RC-22C, Warner Instruments, Hamden, CT) mounted on the stage of an inverted microscope (AxioVert 10, Carl Zeiss, Germany). The preparation was continuously superfused with 37°C Ringer’s solution (TC-344B in-line heater, Warner Instruments) at a rate of 3 ml/min (Peristaltic Pump Minipuls 3, Gilson, Middleton, WI). The chamber PO₂ near the preparation was continuously monitored (Oxy-Micro with PST-1 probe, World Precision Instrument, FL) during the experiment.

Single unit chemoreceptor afferent nerve fiber activity was recorded from the soma of petrosal neurons projecting to the carotid body by placing a suction electrode in the petrosal ganglion. To facilitate unit identification and measurement of conduction time, a glass pipette filled with 1 M NaCl (0.2-MΩ impedance) was placed in the carotid body and a cathodal electrical stimulus (100 µA, 0.1-ms duration; BSI -2, BAK instruments, Rockville, MD) was delivered by a constant current source (BPG-1, BAK instruments) through the pipette to evoke an orthodromic action potential. Electrical stimuli were delivered only until evoked single-unit activity was detected and spontaneous action potentials were displayed on an oscilloscope. The action potential discharge activity recorded from the sensory petrosal neuron was continuously acquired, amplified (MD5, BAK instruments), filtered and digitized at a sample rate of 10 kHz (Digidata 1200, Axon Instruments, Foster City, CA) and stored on a computer (Axoscope, Axon Instruments) for further analyses.

**Chemoreceptor activity recordings.** Chemoreceptor activity was first recorded under control, normoxic conditions (without drug treatment) for 2-3 minutes (superfused with Ringer’s buffer equilibrated with 21% O₂ and 5% CO₂, balanced with N₂, PO₂ ~150 mmHg). Then, the control hypoxic response was
measured for 3-4 minutes by superfusing the preparation with a Ringer's buffer equilibrated with 5% O₂ and 5% CO₂ balanced with N₂, PO₂ ~60 mmHg. After returning to normoxic conditions and stable activity, the preparation was superfused for 10 minutes with normoxic solution containing either suramin or A317491, and the action potential discharge rate was recorded for 2-3 minutes. The inflowing line was then switched to the hypoxic solution containing the same drug for 3-4 minutes. The drug was washed out for 10 minutes and the hypoxia response retested. Only one drug was tested on a given preparation.

Data collection and statistical analyses.

Ventilation. Ventilatory and metabolic variables during normoxia were measured and calculated each minute during the experiment using the IOX software program (Version 1.8.9 EMKA technology, Paris, Fr). For each rat, the last 5 minutes of ventilatory (fR, VT, $\dot{V}E$) and metabolic ($\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}E/\dot{V}O_2$ and $\dot{V}E/\dot{V}CO_2$) data were averaged. A two-way ANOVA (StatView 5.1) using the drug applied (suramin, A317491, vehicle) and age (4-, 7-, 21-day-old rats) as treatment variables was used to test statistical significance. In these groups, the mean value of minute ventilation in vehicle-treated rats was arbitrarily made 100. Minute ventilation of each rat in the vehicle, suramin and A317491 groups was calculated using the following formula: (x = individual value in normoxia/mean value of vehicle group X 100). This calculation was performed for each of age group studied. Values were expressed as mean ± SEM.

For the hypoxic response, respiratory frequency and tidal volume were obtained on a minute-by-minute basis for the 20 minutes of recording. We expressed the response to hypoxia as peak and steady state. The peak response is the average of the highest values observed in two successive minutes during the first ten minutes of recording, and the steady state response is the average of the last five minutes of recording. The peak and steady state respiratory frequency, tidal volume and minute ventilation in response to hypoxia were expressed as percent changes from normoxia.
ANOVAs were used to determine main effects of treatment and age and interactions between treatment and age. Fisher’s Protected Least Significant Difference (PLSD) tests were used for post-hoc testing if ANOVAs yielded a p value <0.05. Values were expressed as mean ± SEM.

**Chemoreceptor activity.** Chemoreceptor activity was analyzed using an event detection program (CLAMPFIT 9, Molecular Devices, Sunnyvale, CA). The action potential occurrence times were converted to frequency (Hz) (Origin 7.5 program, Microcal software, Northampton, MA) and plotted against oxygen concentration of the recording chamber and time to determine the activity under normoxia and hypoxic conditions. Discharge rates were averaged over the entire three-minutes recording under normoxia. In contrast, discharge rates under hypoxia were quantified as the peak value of a three-seconds moving average. Significance was tested using a two-way ANOVA with treatment and age as grouping variables. If a p value <0.05 was reported, post-hoc Fisher’s PLSD tests were performed. Values were expressed as mean ± SEM.
Results

Ventilatory and metabolic effects of purinergic receptor antagonists in developing rats

**Normoxic ventilation:** Suramin - Representative respiratory recordings obtained from P4, P7 and P21 rats at 30 minutes after suramin injection are shown in Fig. 1A, 1B, while ventilatory (fR, VT and minute ventilation) and metabolic variables (O2 consumption, CO2 production, \( \dot{V}_E/\dot{V}O_2 \) and \( \dot{V}_E/\dot{V}CO_2 \)) are presented in Table 1. Compared with vehicle, suramin significantly decreased fR (treatment effect, p=0.002), significantly decreased minute ventilation (treatment effect, p=0.0001), and showed a tendency towards decreased VT (treatment effect, p=0.08) over the four ages studied. No significant interaction between age and treatment was detected for fR (age X treatment, p=0.09) or minute ventilation (age X treatment, p=0.4).

Suramin reduced both oxygen consumption (treatment effect, p=0.03) and CO2 production (treatment effect, p=0.01) in P4, P7, P12 and P21 rats (Table 1) with no significant interaction between age and treatment (age X treatment, p=0.5 and p=0.8 for oxygen consumption and CO2 production, respectively). Neither \( \dot{V}_E/\dot{V}O_2 \) (treatment effect, p=0.9) nor \( \dot{V}_E/\dot{V}CO_2 \) (treatment effect, p=0.7) or body temperature (treatment effect, p=0.5) was reduced by suramin compared with vehicle (Table 1), which likely indicated that ventilation was reduced to accommodate the lower metabolic requirements (i.e., suramin did not induce specific hypoventilation in normoxia).

**A317491** - Considering the results obtained with suramin, we studied the effects of the specific P2X3 antagonist A317491 in P4, P7 and P21 rats. Representative respiratory recordings at each age are shown in Figure 1C, and mean values for respiratory and metabolic variables are presented in Table 2. At each age studied, A317491 significantly decreased fR (treatment effect, p=0.0003), VT (treatment effect, p=0.001) and minute ventilation (treatment effect, p=0.0001) compared with vehicle (Table 2). A317491 also decreased oxygen consumption (treatment effect,
p=0.004) and CO₂ production (treatment effect, p=0.009), but \( \dot{V}_E/\dot{V}O_2 \), \( \dot{V}_E/\dot{V}CO_2 \) and body temperature remained unchanged (Table 2). There was no significant interaction between treatment and age for respiratory frequency (age X treatment, p=0.3), tidal volume (age X treatment, p=0.09) or minute ventilation (age X treatment, p=0.2).

There was a clear treatment effect of the P2X antagonists in normoxia (p=0.0001) but no interaction between age and treatment (p=0.9) (Fig. 2). Furthermore, the effects of A317491 on normoxic minute ventilation were greater than those of suramin on P4, P7 and P21 rats. This difference might be related to the difference in the dose used (3.75-fold more A317491 than suramin) or to different binding capacities of these antagonists.

**Hypoxic response:** *Suramin* - Minute-by-minute ventilation data during hypoxic exposure is presented in Figure 3A, which shows the clear effects of suramin on the hypoxic response for minute ventilation in P7 and P21 rats. Percent changes from normoxia are presented in Figure 4A and 4B, which show that suramin significantly reduced the peak (treatment effect, p=0.01) and steady state responses (treatment effect, p=0.001) for fR but not \( V_T \) (treatment effect, p=0.8 and p=0.6 for peak and steady state, respectively) in P4, P12, P7 and P21 rats. Although the peak minute ventilation was slightly reduced in P4 rats, a significant effect was only obtained in P7-P21 rats (Fig. 4A). A similar result was observed during the steady state response to hypoxia (Fig. 4B). There was no significant interaction between age and treatment for the effects of suramin on both the peak and steady state values for fR (age X treatment, p=0.3 and p=0.09 for peak and steady state, respectively) or for minute ventilation (age X treatment, p=0.9 and p=0.5, respectively). Suramin induced a significant decrease in \( \dot{V}_E/\dot{V}O_2 \) and \( \dot{V}_E/\dot{V}CO_2 \) in P7-P21 rats compared with vehicle (Fig. 4B), which indicated profound hypoventilation relative to metabolic needs in hypoxia, although suramin induced no significant interaction between age and treatment for \( \dot{V}_E/\dot{V}O_2 \) and \( \dot{V}_E/\dot{V}CO_2 \) (p=0.08).
A317491 - Minute ventilation during hypoxic exposure was decreased by A317491 at all ages studied (Fig. 3B) and decreased both the peak and steady state responses relative to vehicle (Fig. 4 A, B) (treatment effect, p=0.02 and p=0.003, respectively). Like suramin, there was no significant interaction between age and treatment for the effects on the peak or the steady state responses of minute ventilation (age X treatment, p=0.7 and p=0.3, respectively). As reported for normoxia, A317491 had a stronger inhibitory effect than suramin at P4, P7 and P21 (Fig. 4A, B). The inhibitory effects of A317491 in hypoxia resulted in a hypoventilation, as shown by the reduced $\dot{V}_E/\dot{V}O_2$ and $\dot{V}_E/\dot{V}CO_2$ in P4, P7 and P21 rats (Fig. 4B).

Carotid body response to purinergic receptor antagonists in developing rats

Chemoreceptor activity in response to suramin: For these experiments, we used 24 carotid body-carotid sinus nerve-petrosal ganglion preparations from P3-4 (n=6), P7-8 (n=5) and P16-21 (n=13) rats. Representative raw activities without (control) and with suramin (100 µM) under normoxic and hypoxic conditions are shown in Figure 5A-C. Compared with control, suramin decreased the chemoreceptor activity for each age group measured under conditions of normoxic isocapnic or hypoxic isocapnic superfusion. Under normoxia, the decreased chemoreceptor activity was significant only in P16-21 (treatment effect, p=0.03, Fig. 5D) rats, but in hypoxia the chemoreceptor response was significantly decreased at all ages (treatment effect, p=0.0001, Fig. 5E). In line with the ventilatory data, the effects of suramin on chemoreceptor activity was not age-dependent (ANOVA, age X treatment, p=0.5 and p=0.6 for normoxia and hypoxia, respectively, Fig. 8A, B). These inhibitory effects of suramin were in line with a previous study using a similar dose that showed a decrease in the frequency of chemoreceptor activity in P19-21 rats without a significant effect on nerve conduction velocity or action potential amplitude (12).

Chemoreceptor activity in response to A317491: For these experiments, we
used 19 carotid body-carotid sinus nerve-petrosal ganglion preparations from P3-4 (n=5), P7-8 (n=5) and P16-21 (n=9) rats.

Because no data are currently available in the literature describing the use of A317491 on peripheral chemoreceptors, we initially tested its specificity on chemoreceptor activity. Figure 6A-C shows an example of the effect of A317491 on tissue prepared from P3-4 rats. The orthodromically evoked action potentials were recorded after an electrical stimulus at S1, S2 and S3 (Fig. 6A) to evaluate the nerve conduction time and action potential amplitude without and with A317491 (Fig. 6C). Neither the nerve conduction time (time lapsed from the stimulus artifact to the arrival of an afferent action potential at the soma of the petrosal neuron) nor the action potential amplitude (the amplitude difference between baseline and the action potential peak) was affected by A317491 (Fig. 6C), suggesting that the drug had no anesthetic effect.

Raw action potential activities without (control) and with A317491 (10 µM) under normoxic and hypoxic conditions are shown in Figure 7A-C. As reported for suramin, the discharge rate was inhibited by A317491 under conditions of normoxic isocapnic or hypoxic isocapnic superfusion (Fig. 7A-C). However, the decrease in discharge rate under normoxic superfusion was not significant due to the high variability in chemoreceptor activity relative to the low discharge frequency. During hypoxia, the discharge rate was significantly lower than control at each age studied (treatment effect, p=0.001, Fig. 7E). In addition, there was no interaction between age and treatment (ANOVA age X treatment, p=0.5 and 0.6, for normoxia and hypoxia, respectively; Fig 8A, B).

Finally, unlike the ventilatory responses (Fig. 2, 4), there was no significant difference between suramin and A317491 with respect to their effects on chemosensory activity in normoxia (treatment effect, p=0.06, Fig. 8A) and hypoxia (treatment effect, p=0.1, Fig. 8B), although the dose of suramin used was 10-fold higher than that of A317491.
Discussion

The primary result from the present study is that antagonism of P2X-(suramin) and P2X3-containing receptors (A317491) exert a significant effect on normoxia and hypoxia-stimulated ventilation across the range of ages studied. These actions are mediated, in part, by peripheral chemoreceptors, which are inhibited by both suramin and A317491. This result is broadly consistent with previous studies that demonstrated a critical role of P2X receptors in sensory transduction at the carotid body in mature animals (38). Furthermore, the effects of antagonists were relatively constant across the range of ages. Thus, despite significant changes in respiratory control in early life, the contribution of P2X receptors to the overall ventilatory level remains relatively constant.

Technical limitations. There are some technical considerations inherent in the present experiments. First, accurate measurement of tidal volume using whole-body plethysmography on very small animals is problematic (30). However, as previously discussed (23, 31), the use of a vehicle group for each age category studied was appropriate because all experiments were performed under similar conditions. Furthermore, rat pups were asleep for 70-80% of the time (4, 8), so we cannot deny the possibility that our recordings were made while the rats were asleep. Interestingly, the results of our in vitro experiments were in line with the plethysmography ventilation recordings. Second, the drugs tested here may have crossed the blood-brain barrier, so the ventilatory effects of the drugs may involve both peripheral and central blockade. Findings on whether suramin may cross (35) or not cross (39) the blood-brain barrier have been inconsistent. Currently available data indicate that A317491 does not cross the blood-brain barrier (45). In addition, the efficiency of the blood-brain barrier changes developmentally (27), so central effects of the drugs may have been present at some ages and not others. This issue could be addressed using chemodenervated rat pups, but this surgery is exceptionally challenging in rats of young ages and is associated with a high mortality rate (40). Interestingly, P4 and P7 rats that received suramin or A317491 showed respiratory patterns under normoxic condition that were similar...
to chemodenervated pups (40). The results of treatment with A317491 or suramin being consistent between the *in vivo* and *in vitro* experiments suggests that the ventilatory results were mostly related to peripheral rather than central activation of purinergic receptors.

Finally, with respect to single-unit chemoreceptor recording, the number of sampled chemoreceptor neurons was relatively small and may not reflect the level of carotid chemoreceptor activity for the entire population. However, this limitation is likely to be minor because there was good agreement in the present experiments between the chemoreceptor recordings and the observed ventilatory changes produced by hypoxia or drug treatment.

**Implication of ATP and purinergic receptors on respiratory control.** A role for ATP acting through purinergic receptors is well established within the mature carotid body. The chemoreceptor afferent fibers within the carotid body show immunoreactivity to P2X<sub>2</sub> and P2X<sub>3</sub> receptors (34). In addition, the messenger RNA for P2X receptors is present in high concentrations in the carotid body and petrosal ganglia, which contain the soma for chemoreceptor afferent neurons (3). ATP is rapidly released from the carotid body during stimulation with hypoxia (10, 16), and antagonism of P2X receptors with suramin or pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonate (PPADS) reduces chemoafferent activity (21, 34, 43) and postsynaptic activity in petrosal neurons co-cultured with carotid body glomus cells (48). Similarly, knockout mice lacking the P2X<sub>2</sub> receptors showed a clear decrease in the carotid sinus nerve chemoreceptor activity and in the ventilatory response to acute hypoxia. These responses are further reduced when both P2X<sub>2</sub> and P2X<sub>3</sub> subunits are deleted in the P2X<sub>2/3</sub> double knockout mice (18, 38). In petrosal neurons, αβ-methylene-ATP (a specific agonist of P2X<sub>3</sub> receptors) produces fast and long-lasting inward depolarizing currents, demonstrating the functional presence of P2X<sub>3</sub> receptors in these neurons (48).

All of these observations suggest an important role of activation of P2X<sub>3</sub> containing receptors in specific neurons to affect the ventilatory and chemoreceptor activity of adult animals. In addition to mediating an excitatory role
at the carotid body, ATP is rapidly released in the brainstem and plays an important role in mediating central processing of respiratory input (18, 19, 41). Purinergic receptors for ATP, present as homomeric and heteromeric forms on cellular membranes (32), have been identified in the nucleus tractus solitarius (19, 24), which receives the central projections of the carotid sinus nerve.

**Purinergic receptors mediate potent tonic drive under normoxia in rat pups.** In rat pups aged P4 to P21, suramin and A317491 inhibited ventilation during normoxia, as previously reported in adult rats (19, 42). The $\dot{V}_E/\dot{V}O_2$ and $\dot{V}_E/\dot{V}CO_2$ were not altered by P2X blockade, indicating that ventilation is reduced in proportion to the reduced oxygen consumption and CO$_2$ production. Thus, blood gases were likely not affected by the drugs. It seems likely that the reduced carotid body activity (as observed in vitro) contributed to this reduction of ventilation, but this assertion requires confirmation. The mechanism by which suramin reduces metabolic rate is not known, but suramin has been shown to decrease the activity of glycolytic enzymes in *Trypanosoma Brucei* (the parasite that causes African sleeping sickness) (14) and reduces respiration in isolated liver mitochondria (6). Whether these effects are also mediated by A317491 is currently unknown.

Across all the ages studied, the reduction in normoxic chemoreceptor activity was approximately 80% (Fig. 8A), a level consistent with that observed in mice lacking the heteromeric forms of P2X$_{2/3}$ receptors. In mice lacking homomeric P2X$_2$ receptors, the decrease in resting activity was reported to be approximately 40% (18). Hence, data obtained here together with those in the literature are in line with the tonic role of ATP and the implication that P2X$_3$-containing receptors regulate ventilation at rest in both newborn and adult rats. The magnitude of the inhibition in minute ventilation and the reduction of chemoreceptor activity by purinergic antagonists were relatively constant over the age range studied (Fig. 2 and Fig. 8A), suggesting that the contribution of ATP receptors to normoxic chemoreceptor activity is constant over development.
Purinergic receptors are involved in hypoxic ventilatory responses in rat pups. Both suramin and A317491 produced a significant reduction in the ventilatory response to acute hypoxia, and this effect was most pronounced between P7 and P21. A317491 produced a significant reduction in ventilation at P4, while suramin produced no statistically significant effect on ventilation in P4 rats. The larger effects in older rats were likely due to the enhanced peripheral chemoreceptor activity following maturation, which largely takes place over the first week of life and continues through the second and third weeks (25). The ventilatory changes were consistent with the large decrease in chemoreceptor activity during hypoxia following treatment with suramin and A317491 (Fig. 5E, 7E). As in normoxia, the relative decrease in chemoreceptor activity following treatment with P2X blockers was relatively constant across ages, suggesting that the role of P2X stimulation is constant across early development. However, although the relative role is constant, the absolute role played by P2X receptors might actually increase with development, as shown by the change in absolute AP discharge rates following suramin or A317491, which were greater at P16-P21 than P3-4 (Fig 5E, 7E). This finding suggests that ATP receptors contribute to the higher spiking activity of the mature organ.

Perspectives. This study clearly demonstrates that P2X receptors are involved in the hypoxic ventilatory response in developing rats and highlights the important role of P2X3 containing receptors. The lack of difference between the effect of suramin and A317491 on chemosensory neuronal activities, together with the lack of age-dependent effects of either drug, are in line with previous data showing no changes in levels of P2X2 or P2X3 receptors in the carotid body and petrosal ganglion during the first two weeks of life in cats (3). Because ventilation and the chemosensory response to hypoxia were not completely abolished by suramin or A317491, other excitatory pathways, such as metabotropic P2Y receptors (29) or other transmitters (2), are necessarily involved. In particular, we have recently demonstrated that cholinergic receptors might be critically involved.
in postnatal maturation of the hypoxic ventilatory response (31). Furthermore, interactions between different receptors might also play a role in the process of carotid body maturation because receptor-receptor interaction has been recently suggested to play a role at rest and in response to hypoxia (46). Finally, how ATP and its receptors interact with the other modulatory factors, such as a persistent Na⁺ current in chemoafferent fibers (15), needs to be better understood.

**Acknowledgements:** This study was supported in part by Téléthon des Étoiles to Dr A. Bairam and NIH grant HL084520 to DF. Donnelly. LM. Niane holds a PhD fellowship from the Respiratory Health Network of FRQS. The authors thank Merlin M. Njoya for his advice on statistical analyses (MSc in biostatistics, CR-CHUQ), Van Diep Doan for technical assistance, and Mélanie Pelletier and Sylvie Viger for animal care.
**Figure Legends**

**Figure 1.** Typical respiratory recordings under normoxia (FiO₂ = 21%) from three different pups at postnatal day 4 (P4), P7 and P21 after a single injection of either vehicle (A, saline), suramin (B) or A317491 (C). These examples were obtained during the last 5 minutes of recording during normoxia. Note that these are flow traces, tidal volume is calculated from the integrated signal. Inspirations correspond to downward deviations below 0.

**Figure 2.** Comparative effects of suramin and A317491 on minute ventilation in developing rats under normoxia. The mean minute ventilation in vehicle-treated rats was arbitrarily assigned a value of 100. Minute ventilation of each rat in vehicle, suramin or A317491 groups was calculated using the following formula: \( x = \frac{\text{individual normoxia}}{\text{mean value of vehicle group}} \times 100 \). This calculation was done for each age group studied. The dose of suramin was 40 mg/kg (IP), and the dose of A317491 was 150 mg/kg (IP). Values were expressed as mean ± SEM. *p<0.05 vs. vehicle (saline); †p<0.05 vs. suramin. P4 (☐: vehicle, n=10; ■: suramin, n=10; □: A317491, n=5); P7 (☐: vehicle, n=6; ■: suramin, n=12; □: A317491, n=6); P21 (☐: vehicle, n=10; ■: suramin, n=11; □: A317491, n=6).

**Figure 3.** Effects of suramin (A) or A317491 (B) on minute-by-minute changes of ventilatory response to hypoxia in P4, P7 and P21 rats. The suramin dose was 40 mg/kg (IP), and the A317491 dose was 150 mg/kg (IP). Figure 1 presents representative tracings from this experimental group. The bar with the * indicates significant difference between vehicle and suramin (A) or A317491 (B) at each point. A) Suramin group: P4 (☐: vehicle, n=10; ■: suramin, n=10); P7 (☐: vehicle, n=10; ■: suramin, n=12); P21 (☐: vehicle, n=14; ■: suramin, n=11). B) A317491 group: P4 (☐: vehicle, n=10; ■: A31749, n=5); P7 (☐: vehicle, n=6; ■: A317491, n=6); P21 (☐: vehicle, n=10; ■: A317491, n=6).

**Figure 4.** Percent changes from normoxia of respiratory frequency, tidal volume
and minute ventilation responses to hypoxia measured at peak (A) and at steady
state (B) after a single injection of vehicle or suramin (40 mg/kg) in P4, P7, P12
and P21 rats or after administration of A317491 (150 mg/kg) in P4, P7 and P21
rats. Percent changes from normoxia of ventilatory equivalent for oxygen and
carbon dioxide responses to hypoxia were only determined at steady state (B).
Values are mean ± SEM. *p<0.05 vs. vehicle (saline). P4 (○: vehicle, n=10; □:
suramin, n=10; ■: A317491, n=6), P7 (○: vehicle, n=6; □: suramin, n=12; ■:
A317491, n=6), P12 (○: vehicle, n=11; ■: suramin, n=10), P21 (○: vehicle, n=10;
■: suramin, n=11; ■: A317491, n=6).

Figure 5. Effects of suramin on chemoreceptor activities in developing rats. A, B,
and C show typical recordings of single-unit chemosensory activities of petrosal
ganglion neurons in response to normoxic-isocapnic and hypoxic-isocapnic
superfusion without (control) and with suramin (100 µM) in developing rats.
Suramin depressed the chemosensory discharge rate in normoxic (D) and hypoxic
(E) superfusion solutions. Please note the difference in the scale between panels
D and E. Values are mean ± SEM. *p<0.05 vs. control. P3-4 (n=6), P7-8 (n=5),
P16-21 (n=13).

Figure 6. (A) Polygraphic recording of experimental run. Top trace: oxygen
tension in the recording chamber during the run. Horizontal bar indicates
superfusion period with A317491. Bottom trace: raw recording of action potential
activity from the petrosal chemoreceptor neuron with time on abscissa.
Stimulations, S1, S2 and S3, indicate the periods where the electrical stimulation
of the carotid body was induced to initiate orthodromic action potentials. (B) Action
potential frequency plotted against time during an experimental run, with A317491
perfusion time indicated by horizontal bar. (C) Overlaid traces during orthodromic
stimulation for S1, S2 and S3. A317491 had no effect on action potential
amplitude and did not change the conduction time, suggesting no direct
anesthetic action on afferent nerve function.
**Figure 7.** Effects of A317491 on chemoreceptors activity in developing rats. A, B, and C show typical recordings of single-unit chemosensory activities of petrosal ganglion neurons in response to normoxic-isocapnic and hypoxic-isocapnic superfusion without (control) and with A317491 (10 µM) in developing rats. A317491 depressed the chemosensory discharge rate in normoxic (D) and hypoxic (E) superfusion solutions. Please note the difference in the scale between panels D and E. Values are mean ± SEM. *p<0.05 vs. control. P3-4 (n=5), P7-8 (n=5), P16-21 (n=9).

**Figure 8.** Normalized chemosensory discharge activity in developing rats. For each age group studied, discharge rates during superfusion with suramin or A317491 were normalized to control (superfusion without drugs) for normoxic (A) and hypoxic (B) conditions.
References:


Figure 2

Minute ventilation (arbitrary units)

Normoxia

- Vehicle
- Suramin
- A317491

* p < 0.05 vs vehicle
† p < 0.05 vs suramin
figure 3

A) Suramin

B) A317491

minute ventilation (ml/min/100g)

Hypoxia duration (min)

P4

P7

P21

Vehicle

Suramin

A317491

Vehicle
Breathing frequency (% changes from normoxia)

Tidal Volume (% changes from normoxia)

Minute ventilation (% changes from normoxia)

VE/VO2 (% changes from normoxia)

VE/VCO2 (% changes from normoxia)

A) Hypoxia response (Peak)

B) Hypoxia response (Steady state)

* p<0.05 vs vehicle
† p<0.05 vs suramin

Vehicle
Suramin
A317491

P4 P7 P12 P21

Figure 4
Suramin (100 µM) modulates the discharge rate in neurons under normoxic and hypoxic conditions. In control conditions, neurons exhibit a baseline discharge rate which is disrupted by exposure to 5% O2 for 20 seconds. Suramin treatment reduces the magnitude of this response.

**Figure 5**

- **A)** P3-4
- **B)** P7-8
- **C)** P16-21

**D) Normoxia**

- Discharge rate (Hz) for control and Suramin treatment in P3-4, P7-8, and P16-21.

**E) Hypoxia**

- Discharge rate (Hz) for control and Suramin treatment in P3-4, P7-8, and P16-21.

*p < 0.05 vs control*
Figure 6

A: 

\[ \text{A317491 (10\mu M)} \]

\[ P_{O_2} \text{ (torr)} \]

Time (min)

S1  S2  S3

B: 

\[ \text{A317491} \]

Frequency (Hz)

Time (min)

C: 

Control (S1)

A317491 (S2)

Wash (S3)

Time (s)
A317491 (10 µM)

Control

5% O₂

20s

A) P3-4

B) P7-8

C) P16-21

D) Normoxia

E) Hypoxia

Discharge rate (Hz)

Discharge rate (Hz)

* p<0.05 vs control
figure 8

A) Normoxia

B) Hypoxia

Normalized activity (%)

Suramin (100µM)

A317491 (10µM)
Table 1. Suramin effect on baseline ventilatory and metabolic parameters in developing rats.

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Drug</th>
<th>n</th>
<th>Weight (g)</th>
<th>Temperature (°C)</th>
<th>fR (breaths/min)</th>
<th>VT (ml/100g)</th>
<th>VE (ml/min/100g)</th>
<th>VO2 (ml/min/100g)</th>
<th>VCO2 (ml/min/100g)</th>
<th>VE / VO2</th>
<th>VE / VCO2</th>
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Body temperature (°C) recorded orally (P4 and P7) and rectally (P12 and P21), Respiratory frequency (fR), tidal volume (VT), minute ventilation (VE), oxygen consumption (VO2), carbon dioxide production (VCO2), oxygen and carbon dioxide convection ratios (VE / VO2 and VE / VCO2) after single i.p injection of vehicle (saline) or suramin (40mg/kg) in 4, 7, 12 and 21 day old rats.

Values are expressed as mean±S.E.M; *p<0.05 vs vehicle.
Table 2. A317491 effect on baseline ventilatory and metabolic parameters in developing rats.

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Drug</th>
<th>n</th>
<th>Weight (g)</th>
<th>Temperature (°C)</th>
<th>fR (breaths/min)</th>
<th>V&lt;sub&gt;T&lt;/sub&gt; (ml/100g)</th>
<th>V&lt;sub&gt;E&lt;/sub&gt; (ml/min/100g)</th>
<th>V&lt;sub&gt;O2&lt;/sub&gt; (ml/min/100g)</th>
<th>V&lt;sub&gt;CO2&lt;/sub&gt; (ml/min/100g)</th>
<th>V&lt;sub&gt;E&lt;/sub&gt; / V&lt;sub&gt;O2&lt;/sub&gt;</th>
<th>V&lt;sub&gt;E&lt;/sub&gt; / V&lt;sub&gt;CO2&lt;/sub&gt;</th>
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<td>Vehicle</td>
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<td>0.86±0.1</td>
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</table>

Body temperature (°C) recorded orally (P4 and P7) and rectally (P21), Respiratory frequency (fR), tidal volume (V<sub>T</sub>), minute ventilation (V<sub>E</sub>), oxygen consumption (V<sub>O2</sub>), carbon dioxide production (V<sub>CO2</sub>), oxygen and carbon dioxide convection ratios (V<sub>E</sub> / V<sub>O2</sub> and V<sub>E</sub> / V<sub>CO2</sub>) after single i.p injection of vehicle (saline) or A317491 (150mg/kg) in 4, 7 and 21 day old rats.

Values are expressed as mean±S.E.M, *p<0.05 vs vehicle.